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TAASTRUP 27 May 1999

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The present invention relates to a continuous antigen reactive T cell line obtained in vitro, a method for preparing an antigen reactive continuous T cell line, a method for preparing a continuous T cell line with cells from a human having a disease associated antigen or a superantigen, a method for treating or preventing a T cell mediated disease by use of a T cell vaccine, T cell vaccines.

Like other normal human somatic cells, T lymphocytes are believed to have a finite in vitro lifespan. However, continuous T lymphocyte cell lines has been established from chronic inflammatory skin diseases when the culture medium is supplemented with IL-2 and IL-4 but without antigen and accessory cells added.

Among the theories concerning the immortalized T cell lines disclosed by Kaltolt et al (1995) the following was suggested: Chromosome abnormalities, faulty selection in thymus, induction by virus, effect of the inflammation itself, loss of the T cell antigen receptor complex or other intrinsic factors as disscussed in the article. This is also supported in the subsequent rewiev of the subject (Effros et al)[11] wherein the chromosomal abnormalities are mentioned as the relevant thesis for escape from the replicative senescence of the T cells.

Surprisingly, it has now been recognized that the immortalisation is obtained due to the presenting of antigen. This is in conflict with the present knowledge, that antigen mediated T cell proliferation are transient as also claimed in Kaltoft et al 1995 [6].

Accordingly, it has now surprisingly been shown that T lymphocytes can be induced to cytokine dependent continuous growth following antigen activation.

This knowledge is important with respect to the possibility of preparing an relevant amount of specific T cell for a T

cell vaccine as the prior main problem with treatment of humans with respect to T cell vaccination is the fact that suitable immortalized T cells having antigen activity has never been expected to be avaible. However, in mice, T cell vaccines has shown great expectations because virus induced immortal cell lines has been effectful.

The antigen reactive T cell lines according to the present invention may further be utilized in the applications disclosed herein.

SUMMARY OF THE INVENTION

Upon allostimulation peripheral blood CD4+ T lymphocytes reproducibly escape from cellular senescence. These IL-2 and IL-4 dependent alloreactive continuous T cell lines show high telomerase activity. Withdrawal of either IL-2 or IL-4 results in cell growth arrest concomitant with down regulation of telomerase activity. When cultured continuously, these CD4+ human T lymphocytes gradually loose expression of CD28.

The concept of cellular senescence is based on the belief that human somatic cells in vitro are constrained by replicative senescence. This phenomenon has been well documented for human fibroblasts [1,2] keratinocytes and hepatocytes [3,4]. Human T lymphocytes from chronic inflammatory skin diseases, however, has been an exception to this dogma, as it has been shown that they may escape from replicative senescence when cultured in a medium supplemented with IL-2 and IL-4 [5,6]. As neither addition of antigen nor antigen presenting cells were required to establish continuous growth, these T lymphocytes seemed to be activated in vivo in such a manner that continuous in vitro growth only required addition of a sufficient cytokine source.

In addition, immune tolerance against the plethora of microorganisms and dietary antigens is essential for normal gut function. However, in Crohn's disease this tolerance seems to

be broken orchestrated by activated cytokine producing T lymphocytes (13,14,15). The establishment of continuous gut T cell lines could facilitate a detailed analysis of subpopulations of T lymphocytes with respect to their phenotype, and cytokine production.

As shown in Example 2, T lymphocytes from a gut biopsy specimen of a patient with Crohn's disease grew continuously in a medium supplemented with interleukin-2 (IL-2) and interleukin-4 (IL-4) but without antigen and accessory cells added. The cell culture developed into a clonal immortal CD4+ T cell line, Gut_I-1. To investigate whether superantigens directly could induce cytokine production in Gut_I-1 cells, four arbitrarily chosen enterotoxins were tested. Upon direct addition of staphylococcus interotoxins A, D and E (SEA, SED and SEE) IFN-γ production was induced in Gut_I-1 cells.

Gut_R-2, a V_β19+ clonal CD4+ T cell line was established from outgrowing gut T lymphocytes by allostimulation in the presence of IL-2 and IL-4. Gut_R-2 constitutively produces IL-10. Upon direct addition of staphylococcus enterotoxin B (SEB) IL-10 production was markedly enhanced together with a significant IFN-γ production.

In conclusion, the results presented here shows that Gut_I-1 represents an inflammatory subpopulation of gut T lymphocytes whereas Gut_R-2 corresponds to a regulatory subpopulation both with cytokine production augmented directly by autopresentation of certain superantigens.

Accordingly, in one embodiment, the present invention also relates identification of novel subpopulation of T cells such as gut T cell lines. Such sub population may devided into inflammatory and regulatory subpopulations, respecively. The inflammatory subpopulation being useful for T cell vaccination as described herein, whereas the regulatory sub population may be used for investigation of the interaction between the various cell types of the tissue of origin.

A chronic inflammation may be maintained by antigen activation of T lymphocytes, therefore, the surprising finding that antigen stimulation could lead to cytokine dependent continuous growth of peripheral blood T cells or T cells from tissue samples would lead to new therepeutic methods for treating inflammatory diseases including autoimmune disease and also cancer.

Several diseases are also believed to be induced by superantigen, including diabetes (IDDM), multiple sclerosis, psoriasis, atopic dermatitis, Inflammatory Bowel Disease, and several other diseases having an inflammatory aspect.

DETAILED DISCLOSURE OF THE INVENTION

T cell vaccination (i.e. immunization with attenuated activated autoreactive T cells/T cell lines) will be an attractive treatment for patients suffering from T cell mediated autoimmune and chronic inflammatoric diseases.

Murine animal models have shown that T cell vaccination gives an efficient protection against these diseases. It is assumed that the disease associated T lymphocytes through activating and attenuating (for example via γ-irradiation) followed by immunizing increase the activity of immune regulatoric T cells which either eliminates or down regulates the pathogenic T cells. The murine T cell lines used in the T cell vaccination have the advantage that they are continuous (i.e. immortal) which imply that a unlimited number of T cells are available for vaccination as well as for laboratory experiments. Human T cells as other human somatic cells are assumed to have a limited ability for division estimated to be 23 cell population doublings (G Pawelec et al.) equivalent to the fact that from one autoreactive T cell clone 2^{23} - 10^{7} T lymphocytes (- 10 mg T lymphocytes) can be propagated. This number is to small for a human based T cell vaccination. Irrespective of the way human T lymphcytes have been stimulated up to the present invention (with antigenes, mitogenes,

phorbolester, etc.) it has not been possible to obtain a sufficient number of cells for a human based T cell vaccination (reviewed in G Pawelec et al.[22]) unless these T cell lines are immortalized with onkogenic virus (like HTLV-1 and Herpes virus Saimiri). Thus, it has been assumed that the limited growth of T cells (like other human normal somatic cells) is an inapplicable biological phenomenon and therefore not a technical problem (as regards the cell culturing). However, according to the present invention, it is shown that T cells with predefined antigen specificity and under certain conditions (high IL-2 + IL-4 concentration) immortalize, and that these immortal T cell lines preserve their antigen recognition. Thus, the system as described selects only for continuous growth of T lymphocytes with a certain antigen specificity and furthermore selects for the antigen specific T cell clone that has the shortest cell population doubling time (i.e. the clone that grows the fastest). Thus, the method obtains antigen specific T cell clones without it being necessary to clone in the conventional way (via a socalled "limiting dilution cloning"). In this way the method as described indicates how immortal antigen specific T cell clones can be generated (without the help of onkogene virus) and besides leads to the new recognition that there is at least one human somatic cell type (i.e. the T lymphcyte) that departs from the dogma that (all) human cells age when being cultured.

In the present context, superantigens are molecules that stimulates a subset of T cells by binding to MHC class II molecules and V_β domains of T-cell receptors, stimulating the activation of T cell expressing particular V_β gene segments.

In the present contect, the term antigen specific and antigen reactive T cells relate to antigen recognition by T cells, leading to partial or full activation of the T cells.

In one embodiment, the present invention relates to a continuous antigen reactive T cell line obtained in vitro by

a) obtaining T cells and antigen presenting cells from a mammal including a human,
b) mixing or contacting said T cells and antigen presenting cells with an antigen for activation of the T cells,
c) and culturing the mixed cells in a medium comprising at least one cytokine.

In a furher embodiment, the present invention relates to a method for preparing an antigen reactive continuous T cell line comprising
a) obtaining T cells and antigen presenting cells from a mammal including a human,
b) mixing/contacting said T cells and antigen presenting cells with an antigen
c) and culturing the mixed cells in a medium comprising at least one cytokine.

In a further aspect, the method relates to the preparation of an antigen reactive continuous T cell line comprising obtaining T cells and antigen presenting cells from a mammal including a human having a disease associated antigen or a superantigen, and culturing the T cells in a medium comprising at least one cytokine. Hereby, the patients own antigen activation of T cells ("in vivo" activated T cells) is utilized for a the preparation of a patient specific T cell line

In an important aspect, the invention relates to a method for treating or preventing a T cell mediated disease in a patient suffering or previously suffering from said T cell mediated disease comprising
a) preparing a continuous disease associated T cell line by use of T cells obtained from said patient and culturing said T cell with at least one cytokine,
b) preparing a T cell vaccine from the continuous T cell line by activating and attenuation of such continuous T cell line
c) administering said T cell vaccine to the patient.

In a further important aspect, the invention relates to a T

cell vaccine prepared from T cells from a continuous T cell line obtained by culturing T cells isolated from a patient suffering from a T cell mediated disease in a medium supplemented with at least one cytokine.

The T cells and antigen presenting cells are for instance isolated from the mammal as a biopsy or a cell sample including a blood sample.

In a still further aspect, the present invention relates to a method for preparing continous antigen activated T cell line comprising

a) obtaining a cell sample from a patient

b) culturing the cells in a medium comprising at least one cytokine

c) separating activated T cell

d) stimulation of said separated activated T cell with an allogen

e) further culturing the allostimulated T cell in a medium comprising at least one cytokine for the production of a continues cell line.

The separating activating T cell is i.e CD69+, and with respect to the stimulation of said separated activated T cell with an allogen, this antigen specifik T cells often show alloreactivity. CD69+ may be a suitbable marker as it is expressed rather early following activation (14-16h). Accordingly, by selection for CD69+ cells (CD4+) after about 24 h inflammatory T cells may be obtained. On the contrary, if selection is performed after a longer period, the regulatory T cells have become activated by the inflammatory cells and thus they also express CD69+. Accordingly, the difference in time and the dynamic in the immunological reaction may be exploited.

By this method a high number of the desired specific cell line is obtained, as T cells which are not activated are eliminated. The existence of the relevant group of activated

T lymphocytes are normally only 1-2% of the cell population, and has not previously been recognized by conventional detection systems, such as by flow cytometry, as this amount of cells are below the detection limit within such systems.

Dependent of the specific disease or purpose, the biopsy may be obtained from a healthy person or from a patient suffering from a T cell mediated disease. With respect to the latter, again the sample may be obtained from a site or organ associated with said disease or when appropriate from a site or organ not afflicted by said disease, depending whether a test of the activity of the disease is detected, or whether a disease specific reactive T cell line is desired.

The T cells may be isolated from a patient suffering from a disease associated with the skin or in a surprising aspect from the intestines such as with an inflammatory bowel disease.

The present invention is believed to be useful for any disease being a chronic inflammatory disease or autoimmune disease. However, as many malignant diseases are associated with chronic inflammation or autoimmune disease or is associated wiht abnormalaties relating to an altered T cell function or cytokine imbalance also malignant disease (cancer) is relevant diseases according to the invention.

Examples of autoimmune disease include arthritis rheumatoides, autoimmune hepatitis, arthitis rheumatoides, autoimmune anæemia hæmolytica, anaemia perniciosa, aphtae, aspermiogenese, Behcets syndrome, primary biliary cirrhosis, chorioiditis, insulin dependent diabetes mellitus, encepalomyelitis allergica, endophthalmitis phacoanaphylactica, Goodpastures syndrom, Graves disease, Hashimotos disease, myastenia gravis, ophtalmia sympathica, orchits granulomatosa, pancreatitis, pemphigus vulgaris, polyradiculitis Qeervains gout, scerosis disseminata, idiopatic thrombocytopeni and many other disease kwown for the skilled person and disclosed in

handbooks of medicine.

Also chronic viral diseases fall within the scope of relevant diseases according to the present invention and includes infection with Hepatitis B and Hepatitis C virus.

Disease of the gastrointestinal lumem are an important and surprising target for the present invention and inlude conditions located in the oesophagus such as:
Oesophagitis, defined as inflammation of the oesophagus.
Conditions located in the stomach such as:
Antral gastritis, defined as inflammation affecting the antrum of the stomach. Non-specific chronic gastritis, defined as chronic inflammation of the stomach of unknown etiology. Polypous gastritis. Pseudomembranous gastritis, defined as a variety of gastritis in which a false membrane occurs in patches within the stomach.

In addition, a surprising embodiment is the application of the present invention is with respect to the intestines as the intestine have a high level of gamma/δ positive cells which is activated to killer cell by the presence of cytokines such as especially IL-2 (lymfokine activated killer cells). (Kaltoft 1994 and Kaldtoft 1995) [5,6].

Conditions located in the duodenum such as:
Duodenitis, defined as inflammation of the duodenum
Conditions located in the jejunum, ileum colon and rectum such as:
Jejunitis, defined as inflammation of the jejunum.
Ileitis, defined as inflammation of the ileum.
Colitis, defined as inflammation of the colon.
Diverticulitis, defined as inflammation of a diverticulum.
Proctitis, defined as inflammation of the rectum.
Mb.Crohn, defined as chronic granulomatous inflammatory disease involving any part of the gastrointestinal tract from mouth to anus of autoimmune etiology.
Ulcerative colitis, defined as chronic recurrent ulceration

of the colon of autoimmune etiology.

Autoimmune conditions, defined as conditions characterized by a specific humoral or cell-mediated immune response against constituents of the body's own tissues such as:
Mb.Crohn, as defined above.
Ulcerative colitis including proctitis, as defined above.
Non tropical sprue or celiac disease, defined as malabsorption syndrome precipitated by ingestion of glutencontaining foods, it is generally believed that autoimmunity plays a major role in the etiology.

Premalignant or malignant diseases such as:
Polypi, defined as solitary of multiple polyps of the digestive tract. Polyposis familiaris, defined as multiple adenomatous polyps with high malignant potential, lining the mucous membrane of the intestine, particularly the colon.
Cancer oesophagi, defined as cancer located in oesophagus.
Cancer ventriculi, defined as cancer located in the stomach.
Cancer duodeni, defined as cancer located in duodenum.
Cancer jejuni, defined as cancer located in jenunum.
Cancer ilei, defined as cancer located in ileum.
Cancer coli, defined as cancer located in colon.
Cancer recti defined as cancer located in rectum.
Cancer analis defined as cancer located in anus.

For the treatment of cancer, according to the present invention, periferal blood cells from patient and attenuated tumor cells from the patient are mixed, cytokines are added and thereby cytotox cells are produced, cultures according to the invention, characterized with respect to phenotype and function. For the treatment of cancer CD8 and/or CD4 cells are preferred, however any other cell capable of killing the specfic cancer cell may by obtained. The killer cells may thereby be produced in a large quantity. Before administering (transfusioin) such killer cells to the patient, the cells are preferably irradiated with gamma rays for the purpose of interrupting cell division, thus leaving the functional

capacity intact (killer activity).

This procedure has been shown to be able to generate cytotoxic continous T cells from two patients with Cutaneus T cell lymphoma CTCL.

It is belived that for the malignant melanom having tumor-infiltating lymphocytes within the tumor, the present invention would be of special interest.

The T cell vaccines according to the present invention are conventionally administered parenterally, by injection as a transfuion, a subcutaneously or a intramuscularly injection.

In another aspect, the invention provides a method of treating and/or preventing a disease preferable a disease as mentioned in the present application such as a chronic inflammation or an autoimmune disease, said method comprising administering to a patient in need thereof a therapeutically or prophylactically effective amount of a T cell vaccine

The at least one cytokines are selected for the group of cytokines known in the art, preferable combinations of IL-2, Il-4, L-7 and IL-9 and IL-15. The preferred combination is of IL-2 and IL-4.

A common feature of the receptor for the cytokine mentioned is the gamma-chain. It is belived that with respect to IL-2, and with the concentrations mentioned, the α-chain of the cytokine receptor is not mandatory for tranduction of the signal in T cells already activated, possible as the transduction of the signal already has a strength for inducing cell division. IL-2 and IL-4 cell increase division and decrease apoptosis and are therefore belived to be involved in the specific balance promoting the immortalization of the cell lines.

The cytokines IL-7 and IL-9 are likely candidates for substi-

tution with IL-4. And IL-15 for IL-2.

The cytokines are preferable used in concentrations of from 500-5000 U/ml preferable 1000-2000 U/ml such as for IL-2, and concentrations of 200-1000, such as about 500 U/ml for IL-4. However it should be noted that the upper limit is not critical, as excess of cytokines are desired.

In a still further embodiment, the present invention relates to a method for a diagnostic test indicating disease aktivity comprising isolating a tissue sample representative for said disease, measuring production of IFN-gamma and/or IL 10 from the sample in a primary culture and aligning said value with a fixed value for said patient.

The present invention may also be utilized in a method for designing a functionnally continouous T cell line, i.e a T cell line with a prespecified antigen recognition associated with T cell function. In a still further embodiment the immortalized T cell line may be used for the standardizing of a T cell mediated assay such as for cytokine detection and for screening for immune antagonists (biological response modifiers).

The immortalized cell lines according to the present invention have a lifespan of at least 40 PD (population doublings), such as at least 60 PD, preferable at least 100 PD, more preferred at least 150 PD, such as at least 200 PD.

In a preferred embodiment, the cytokine profile of the immortalized T cell lines is not substantially altered during the at least 40 PD, in a preferred embodiment the cytokine profile is not substantially altered during the at least 60 PD, such as during the at least 100 PD, more preferred, the cytokine profile is not substantially altered during the at least 150 PD, such as during the at least 200 PD.

By "not substantially altered" is meant that the cytokine

pattern is neither functionally or structurally altered in such a way that the overall effectiveness of the cytokines are substantial different from a the cytokines produced by the initial cell.

In som cases, the inflammatoric IFN-γ producing CD4+ cells are most likely to be superantigen activated, and furhtermore, the substantial part of these may also show alloreactivity. Thsi implies that the cell lines invention besides the native antigen also can be activated by alloantigen.

For example, as shown herein allostimulation of cells from a patient suffering form Crohn's disease gives rise to the Gut_R-2 IL-10 producing cell line which does not recognize the alloantigen with which it was stimulated.

LEGENDS TO FIGURES

Figure 1. Shows the number of cell population doublings, PD, of three PBMC cultures grown in medium with IL-2 + IL-4 alone (left) or with allostimulation in the presence of IL-2+IL-4 (right).

Figure 2. Shows telomerase activity at 100 PD of a continuous peripheral blood activated CD4+ cell line (Act-1) cultured with IL-2 + IL-4, IL-2 or IL-4 as indicated. For comparison, telomerase activity of the leukemic cell line Se-Ax, cultured with IL-2 alone, is also shown.

Figure 3. Shows CD28 expression of the continuous peripheral blood derived CD4+ cell line Act-1 at PD 60 and 150 compared with CD4 and CD8 expression at PD 150 (Flow cytometric analysis).

Figure 4. Shows CD28 expression at different PD of the clonal T cell line My-La, 46,XY,i(18q). Also shown is CD4 and $V_\beta 18$ expression at the different PD and CD8 expression at PD 200 (Flow cytometric analysis).

Figure 5. Shows the phenotype in the growing primary T cell culture from which Gut_I-1 is derived (Example 2)(Flow cytometric analysis).

Figure 6. Shows the phenotype of Gut_R-2 in Example 2 (Flow cytometric analysis).

Figure 7. Shows the phenotype in the growing primary T cell culture from which Gut_I-1 is derived (Example 2)(Flow cytometric analysis).

Figure 8. Shows the karyotype $47,XX_I,+2,t(1;1)$ of Gut_I-1.

Figure 9. Shows the karyotype $45,XY_I,-20,add(1)(p36)$ of Gut_R-2.

EXAMPLE 1

Derivation of finite and continuous peripheral blood T cell lines

Peripheral blood mononuclear cells (PBMC) from 3 healthy donors were isolated by standard Ficoll-Isopaque gradient centrifugation. The PBMC were resuspended at 5 x 10^5 cells/ml in 90% RPMI 1640, 10% human AB serum, 1000 u/ml IL-2 and 500u/ml IL-4 with antibiotics as described (5). To access whether longevity of cultured PBMC is dependent on in vitro activation, PBMC were cultured in the above medium alone or with additional alloactivation. 5 x 10^6 PBMC were stimulated with the heavily γ-irradiated (60 Gy) Psor-2 cell line at a 5:1 ratio. The Psor-2 cell line is a continuous T cell line established from a skin biopsy specimen of a patient with psoriasis vulgaris by culturing the skin specimen in the medium mentioned above (6).

Estimation of CD28 expression as a function of cell population doublings

Monoclonal antibodies against CD3, CD4, CD8, CD28, and CD56 were purchased from PharMingen. An α/β T cell receptor sub-

family antibody against $V_\beta 18$ was obtained from Immunotech. An indirect immunofluorescence technique was applied to label the cells as previously described (5). Allostimulated continuously growing peripheral blood T cell lines were cryopreserved for each 10 PD. Cells cryopreserved at different PD were then thawed, cultured for 4 days and analyzed for CD28 expression by flow cytometry. CD4 and CD8 expression served as positive and negative controls, respectively. For each antibody 2 x 10^4 cells were analyzed (FACS Calibur, Becton Dickinson). Fluorescence microscopy was also applied to evaluate the stainings.

A clonal CD4+, $V_\beta 18$+ T cell line My-La, 46,XY,i(18q) (7,8) cultured with 1000 u/ml IL-2 and 500 u/ml IL-4 was also analyzed for CD28 expression at different PD.

Other methods

Cells were found to be free of mycoplasma by the Hoechst staining test. Telomerase activity of 10^3 cells was determined by the TRAPeze Telomerase Detection Kit as described by the manufacturer (Oncor).

Growth of peripheral blood cells with and without allostimulation

PBMC from the 3 healthy donors proliferated between 1 to 3 PD when cultured in the cytokine supplemented medium alone (Fig 1) in agreement with previously published data showing that peripheral blood cells proliferate only transiently when stimulated with a combination of IL-2 + IL-4 (5,6). However, when PBMC were allostimulated once with the Psor-2 cell line in the presence of a high concentration of IL-2 and IL-4, T cells as well as non T cells (preferentially CD3-, CD56+) proliferated vigorously during the first 4 to 6 weeks.

After approximately 50 PD only CD4+ T cell grew in the cytokine based medium. All three CD4+ allostimulated T cell lines have proliferated beyond 150 PD with a PD-time of 30 to 36 hrs (Fig 1). This corresponds to an increase in cell numbers

of $2^{150} \sim 10^{45}$-fold. As allostimulated peripheral blood T lymphocytes have been estimated to have a limited in vitro lifespan of 23 ± 7 PD (9), the allostimulated CD4+ cell lines reported here can be considered continuous, effectively having an unlimited replication capacity.

So far, the three continuous peripheral blood derived CD4+ cell lines show no sign of growth exhaustion and at PD 150 still retain alloreactivity (results not shown).

Cytokine dependent continuous T cell lines have cytokine dependent telomerase activity

Continuous cell lines are expected to have telomerase activity. When cultured in the presence of both IL-2 and IL-4 in vitro activated peripheral blood CD4+ T cells show high telomerase activity (Fig 2) comparable to that of a leukemic cell line Se-Ax (10), established form a patient with Sezary syndrome. Withdrawal of either IL-2 or IL-4 results in growth arrest. After withdrawal of IL-4, a 100 PD cell culture cease proliferating after 14 to 21 days. Withdrawal of IL-2 results in cell growth arrest between 6 to 9 days. As shown in Fig 2 telomerase activity in IL-2 or IL-4 starved cells is severely reduced. The results indicate that simultaneous presence of IL-2 and IL-4 regulates both growth and telomerase activity in these T cell lines.

CD28 expression correlates inversely with cell population doublings

Allostimulated PBMC cultured in the cytokine supplemented medium became pure CD4+ cell lines after approximately 50 to 60 PD. CD28 expression of one such CD4+ cell line, Act-1, at PD 60 and PD 150 is presented in Fig 3. CD28 expression is clearly detectable at PD 60 but absent at PD 150. A gradual decline in expression of CD28 between PD 60 and PD 150 could be observed (results not shown).

To investigate whether the culture system preferentially expands preexisting CD28 negative CD4+ cells or whether CD28

could serve as a mitotic clock in individual T cells a clonal CD4+, $V_{\beta}18+$ T cell line established from an inflammatory skin biopsy specimen (7,8) was investigated for CD28 expression. As shown in Fig 4 CD28 expression of this T cell clone (My-La, 46,XY,i(18q)) decreases gradually with cell population doublings being present at PD 40 and completely absent at PD 200. However, CD4+ expression is compatible at PD 40 and PD 200. These findings are in agreement with data obtained from finite CD4+ T cell lines (11) showing downregulation of CD28, but not complete loss of CD28 expression with increasing PD. The results presented here show that CD28 expression correlates inversely with cell population doublings and indicates that CD28 expression can serve as a mitotic clock at the clonal level.

The results show that alloactivation with the continuous psoriatic T cell line Psor-2 can efficiently prime allogeneic CD4+ peripheral blood T cells to cytokine dependent continuous growth. These cytokine-driven peripheral blood derived CD4+ T cell lines show IL-2 and IL-4 dependent telomerase activity, and they gradually loose CD28 expression with increasing cell population doublings.

Conclusion

Contrary to other normal human somatic cells T lymphocytes can in vitro like in vivo be activated to continuous cytokine driven growth. The results presented here raises the possibility of generating an unlimited number of T cells with predefined specificity. Such immortal T cell lines may be useful for several applications, for instance for standardization of T cell mediated biological assays and for generating sufficient numbers of autoimmune T cells for human T cell vaccination.

Example 2

Superantigen directly augment the cytokine production of two novel continuous Gut-derived T cell lines from patients with

Crohn's disease

Interferon γ (IFN-γ) producing CD4+ T lymphocytes have been implicated with progression of Crohn's disease whereas inter-leukin-10 (IL-10) producing CD4+ T lymphocytes are thought to down regulate disease activity.

In the following it is investigated whether a newly devised cell culture protocol could select for continuous clonal CD4+ T cell lines producing either IFN-γ or IL-10.

Biopsy specimens

At least eight colonic biopsies were obtained from affected mucosa of two patients. The biopsies were examined for histo-pathological changes and a diagnosis of Crohn's disease was established according to clinical, radiological and histopathological data.

In each patient, two additional biopsies were taken for in vitro culture of T cells. The Gut_I-1 T cell clone was estab-lished from a patient undergoing ciclosporine treatment with a CDAI index of 296 whereas the patient from whom Gut_R-2 derived had a CDAI index of 155. The study was approved by the local ethic committee.

Cell culture

The two biopsies were washed twice in sterile PBS and once in the growth medium. The growth medium consisted of 90% RPMI 1640 10% human AB serum. 100 U/ml penicillin G 100 μg/ml streptomycin (basal medium, BM) supplemented with 2000 u/ml IL-2 and 500 u/ml IL-4 (complete medium). The T lymphocytes were initially expanded in 5 ml complete medium and when cell density reached 1.5 x 10^6/ml, the culture was split at a 1:2 ratio.

T cells of the primary cultures from which Gut_R-2 derived were allostimulated with the heavily γ-irradiated (60Gy) leukemic cell line Se-Ax at a 5:1 ratio. The continuous Se-Ax cell line was established from a patient with Sezary syndrome

(10).

Phenotyping

Phenotyping. Monoclonal antibodies against CD3 (OKT3), CD4 (OKT4), CD8 (OKT8) and CD25 were obtained from hybridomas from American Type Culture Collection (ATCC). Monoclonal antibodies against CD45RO and HLA-DR were purchased from PharMingen. Monoclonal antibodies against TCR-1 (TCRγ/δ), TCR-2 (TCRα/β) and α/β T cell receptor subfamily antibodies against V_β 1, V_β 2, V_β 3, V_β 5.1, V_β 5.2, V_β 5.3, V_β 7, V_β 8, V_β 9, V_β 11, V_β 12, V_β 13.1, V_β 13.6, V_β 14, V_β 16, V_β 17, V_β 18, V_β 19, V_β 20, V_β 21.3, V_β 22 and V_β 23 were obtained through Coulter. An indirect immunofluorescence technique was applied to label the cells as previously described (5). 2 x 10^4 events were analysed by flow cytometry (FACS Calibur, Becton Dickinson) and debris and aggregates were excluded by gating. Fluorescence microscopy was also applied to evaluate the stainings.

Stimulation of cells. Cells cultured in complete medium were washed twice with RPMI 1640 in order to eliminate residual cytokines. They were then resuspended in basal medium with IL-2 or complete medium at 10^6/ml. Cells were then stimulated either with 10 μg/ml monoclonal antibodies against CD3 or with staphylococcus enterotoxins A, B, D and E at a concentrations of 1 μg/ml (obtained from Toxin Technology Madison, WI).

Cytokine determination. Supernatant of stimulated cells and cells cultured in basal medium with IL-2 or complete medium was harvested after 24 or 48 hours. Cytokine matched antibody pairs for determination of IFN-γ IL-4, IL-10 and tumour necrosis factor (TNF-α) were obtained from Endogen. The detecting antibodies were all biotinylated. A time resolved fluorometric assay applying Europium labelled streptavidin and a Delphia 1234 fluorometer was used to determine the cytokine contents as described by the manufacturer (Wallac) As the cell culture medium contained human serum cytokine

concentrations below 100 pg/ml were not considered to be associated with cytokine producing T cells. The data were analysed by a computer programme (Biosoft, Assay Zap).

Other methods. Cells were found to be free of mycoplasma by the Hoechst staining test. Karyotyping with Q banding followed standard procedures. The karyotypes were established according to the International System for Human Cytogenetic Nomenclature (ISCN) (1985).

RESULTS

Establishment, phenotype and constitutive cytokine production of Gut_R-2

When placed in the complete medium, lymphocytes migrated from the biopsy specimens and proliferation was evident within a week. After approximately two weeks the cell culture had expanded to more than 50×10^6 cells. The phenotype of this culture is shown in Fig 1. Both TCR-1 and TCR-2 as well as CD4+ and CD8+ T cells that are present in situ (17) are expanded in the cell culture medium. The TCR-2 population was oligo- or polyclonal as evidenced by their reaction with several V_β subfamily antibodies. A positive staining with a V_β subfamily antibody ranged from 0.2% to 8% (results not shown). The activation marker CD25 is only partially expressed in the growing T cell culture (Fig 5) and another activation marker HLA-DR differs widely in expression among individual T cells. At this stage, the culture was split in two, half of the cells were cultured with additional allostimulation, the other half was cultured in the complete medium alone. Cells kept in complete medium without allostimulation developed into a finite cell culture dominated by CD8+ T cells (results not shown). The allostimulated culture initially also increased the percentage of CD8+ cells. However, after a period with no apparent cell number increase, CD4+ T cells started to proliferate continuously. This CD4+ T cell line Gut_R-2 has proliferated beyond 250 cell population doublings (PD) with a PD time of approximately 36 hrs. As

allostimulated T cell lines have been reported to have a finite lifespan of 23±7 PDs, Gut_R-2 can be considered immortal effectively having an unlimited replicative capacity. At PD ~ 150 Gut_R-2 became independent of IL-4 for continued growth. The phenotype of the continuous Gut_R-2 cell line is presented in Fig 6. Among the V_β subfamily antibodies tested Gut_R-2 only expresses the $V_\beta19$ subfamily of the TCR-2 complex indicating that Gut_R-2 is a clone. This assumption was confirmed by karyotyping as Gut_R-2 after approximately 125 PD developed a clonal chromosome aberration observed in all metaphases (Fig 9). Thus, also by cytogenetic criteria the $V_\beta19$+ Gut-2 cell line is a clonal T cell line. Comparison of Fig 5 and Fig 6 shows that clonal Gut_R-2 CD4+ T cell line develops from $V_\beta19$+ T cells that comprise less than 2% of the T cells in the primary culture. As shown in Table I the $V_\beta19$+ clonal Gut_R-2 T cell line constitutively produces IL-10 in basal medium with IL-2 (and also in complete medium), but without additional stimulation. IL-10 concentrations have been measured over a time period of four months corresponding to an increase in cell numbers of approximately $2^{80} \sim 10^{24}$ - fold.

Establishment, phenotype and karyotype of Gut_I-1

Within ten days lymphocytes from the gut biopsy specimens from which Gut_I-1 derived had expanded to more than 50 x 10^6 cells with a phenotype distribution similar to that shown in Fig 5 (results not shown). Upon culture in the cytokine based medium, but without antigen and accessory cells added, CD4+ T cells continued to expand, and within 20 PD a pure CD4+ T cell line evolved that have proliferated beyond 300 PD with a PD time of approximately 30 hrs. Thus, this cell line Gut_I-1 can be considered continuous. The phenotype of Gut_I-1 at PD 150 is presented in Fig 7 and, as shown, it has markers compatible with mature memory CD4+ T cells. At PD ~ 100 Gut_I-1 developed a clonal chromosome aberration as shown in Fig 8 and like Gut_R-2, Gut_I-1 is also a continuous clonal CD4+ cell line. By phenotyping non of the available subfamily V_β specific antibodies reacted with Gut_I-1. Unlike Gut_R-2 con-

stitutive cytokine production was not detectable in Gut_I-1 cells.

Superantigens directly induce cytokine production in Gut_I-1 cells and augment cytokine production in Gut_R-2 cells

As Gut_I-1 (and Gut_R-2) expresses major histocompatibility complex class II (MHC class II) antigens that are high affinity receptors for several superantigens, we investigated whether these cell lines could somehow autopresent superantigens. Four arbitrarily chosen superantigens SEA, SEB, SED and SEE were tested for their ability to induce cytokine production in Gut_I-1 cells (Table 2). As shown, soluble antibody against CD3 (OKT3) in the presence of IL-2 and IL-4 could not induce detectable cytokine production whereas SEA, SED and SEE induced IFN-γ production.

Similarly, the four superantigens were tested for their ability to alter the cytokine production of Gut_R-2 cells. As shown in Table 3, SEB induced high levels of IFN-γ production and also significantly augmented IL-10 production in Gut_R-2 cells. As SEB activation is selectively induced in T cells bearing V_β3,12,14,15,19 and 20 (19), the results presented in Table III indicate that Gut_R-2 autopresent SEB as classical antigen presenting cells.

DISCUSSION

It has been suggested that the normal tolerance to commensal intestinal bacterial antigens or superantigens is broken in Crohn's disease (13,14,15,20). Activated CD4+ T lymphocytes secreting IFN-γ, thereby activating monocytes/macrophages to enhanced TFN-α production has been implicated in maintenance of Crohn's disease (21).

Gut_1-1 is an inflammatory CD4+ T cell clone established from a gut biopsy specimen without addition of mitogen, antigen and accessory cells. It is thus likely that Gut_1-1 was activated in vivo to cytokine driven growth in vitro. This assumption is compatible with the notion that inflammatory T

cells are highly activated in Chron's disease. It should be noted that the cell culture system selects for the fastest growing T cell clone implicating that several T cell clones with properties like Gut_I-1 exist in the inflamed gut mucosa. The V_β subfamily specificity of Gut_I-1 could not be determined by phenotyping excluding the possibility of preselecting a superantigen that could optimally induce IFN-γ production. However, Gut_I-1 responded by direct addition of SEA, SED and SEE with IFN-γ production indicating that Gut_I-1 can autopresent superantigens. Thus, IFN-γ production by Gut_I-1 cells does not necessarily require a specific antigen presented by antigen presenting cells. If this property is also reflected in vivo, no specific microbial agent may be essential for the inflammatory response. Furthermore, inflammatory T cells bypassing the classical antigen presentation could aggravate a chronic inflammation.

Gut_R-2 is a CD4+ V_β19+ cell clone established by allostimulation of outgrowing gut T lymphocytes. During a period of nine months without allostimulation (150 PD) the clonal Gut_R-2 cell line has constitutively produced IL-10.

As Gut_R-2 expresses both high affinity receptors for SEB (MHC class II), and a SEB responsive V_β chain (10) direct addition of SEB to Gut_R-2 results in a dramatic IL-10 and IFN-γ production. The cytokine production of activated Gut_R-2 cells thus resembles a recently described regulatory CD4+ T cell subset (22).

It is intriguing to speculate that regulatory T cells like Gut_R-2 with constitutive IL-10 production independent of direct antigen activation may contribute to normal gut tolerance. Gut_R-2 shows as mentioned above some properties with a newly described regulatory IL-10 producing CD4+ T lymphocyte population (22). However, Gut_R-2 differs from this subpopulation by constitutive non antigen mediated IL-10 production and by its continuous growth.

An advantage of the cell culture system described here for gut T cell clones is that their continuous growth gives rise to an unlimited number of T cells. Such immortal T cell clones may be useful for testing biological response modifiers, and inflammatory T cell clones like Gut_I-1 could provide the basis for a T cell vaccination of patients with Crohn's disease.

Table 1: *Average cytokine production (pg/ml/ 10^6 T-cells) of five different experiments between PD 150 to PD 225 of continuous growing GUT_R-2 cells.*

IL- 4	IFN-γ	IL- 10	TNF-α
<100	25[illegible]	2460 (1887-3033)	<100

Cells in basal medium with IL-2. 95% confidence intervals in parenthesis.

Table 2: *Cytokine production (pg/ml/10^6 T-cells) in GUT_I-1 after stimulation with superantigens (at PD 120)*

GUTI-1	TNF - α	IFN-γ	IL-10
complete medium	< 100	< 100	< 100
+antibody against CD3	< 100	< 100	< 100
+SEA	< 100	1990 (1817-2163)	< 100

+SEB	< 100	290 (164-416)	< 100
+SED	< 100	1500 (1432-1568)	< 100
+SEE	< 100	2070 (1910-2230)	< 100

95% confidence intervals in parenthesis.

Table 3: *Cytokine production (pg/ml/10^6 T-cells) in GUT_R-2 after stimulation with superantigens (at PD 150)*

GUTR-2	TNF-α	IFN-γ	IL-10
complete medium	< 100	< 100	2850 (2679-3021)
+SEA	< 100	< 100	2840 (2738-2942)
+SEB	< 100	>25000	>25000
+SED	< 100	470 (453-487)	5130 (4899-5361)
+SEE	< 100	< 100	5080 (4613-5867)

95% confidence intervals in parenthesis.

EXAMPLE 3

EXAMPLES OF T CELL VACCINATION

A. Multiple sclerosis (MS)

1. A convenient amount, for example 50 ml, blood in heparin is drawn from a patient with MS.

2. The mononuclear cells of the blood that, other than lymphocytes, contain antigen presenting cells (APC) are isolated by a standard Ficoll-Isopaque gradient hydro-extracting.

3. The cells are disseminated in for example five culturing bottles in the medium consisting of 90% RPMI 1640, 10% human AB serum, antibiotic as well as 1000 u/ml IL-2 and 500 u/ml IL-4. If convenient other cytokines as GM-CSF and TNF-α can be added to the bottles to increase/promote the maturing of the dendritic cells with a strong antigen presenting function.

   At this stage, a selection for antigen activated (i.e. CD69+) may be included.

4. On day 0 antigen, in this case myelin, that the patient's autoreactive T cells react against, is added to one of the bottles. Instead of myelin components of the myelin can be added such as myelin basic protein or proteolipid protein or immune dominating epitopes deriving from these proteins.

5. This addition of antigen is repeated in the next bottle for example on day 2 and the procedure is continued with the other bottles with an interval of a couple of days.

6. Subsequently, the cells are propagated in the IL-2 +

IL-4 containing medium. Notice that if "only" the life of the T lymphocytes can be increased from 23 PD to 60 PD instead of 10^7 cells you will have/get 2^{60} - 10^{18} cells, the equivalent of 1000 tons of cells, which will be sufficient to continue all further experiments and vaccination. In case the T cells apparently does not have the expected ability for growth the antigen stimulation can be repeated, and furthermore co-stimulation with for example phorbolester or mitogen-stimulation may be tried to increase the growth potential.

7. The T cells are tested for their antigen specificity and will after activating and attenuating be ready for T cell vaccination.

B. Insulin dependent diabetes

The same procedure as for A can be used, if only the antigen is for example glutamin acid decarboxylase (GAD)-65, GAD-67, insulin, or heat shock protein 60 (Hsp60).

C. Chron's disease

Chron's disease is a multifactorially conditioned chronic inflammatoric intestinal disease where the normal tolerance of the immune system to the microbial intestinal flora is broken. Here the immune reactive T cell clones (for T cell vaccination) against the microbial flora can be brought about in the following way:

From a intestinal biopsy the aerob as well as the anaerob bacteria are cultured. After the culturing they are sonicised and can now be used as antigen/super antigen. Subsequently the biopsy is washed in a antibiotic-containing medium, and within 14 days the T lympohcytes from the biopsy can be propagated in large number (>50 x 10^6) in an IL-2 + IL-4-containing medium (unpublished observations). Antigen presenting

cells are obtained by ficoll separation of the patient's blood cells, and antigen specific/super antigen specific continuous intestinal T cell clones can now be propagated by adding antigen and γ-irradiated antigen presenting cells to the intestinal biopsy T cells.

An analogous strategy can be used for patients with ulcerosa colitis.

Note that for procedure **A** and **B** as well as for procedure **C** the vaccination is individual (depending on the type of tissue), i.e. it has to be the patient's own cells that are used. Besides, note that T cell vaccination primarily has been intended for persons that are already affected by diseases.

In a futher alternative according to the invention in general, the biopsy or cell sample is cultured comprising IL-2 and IL-4 to enriche for activated T cells, and the activated T cells is isolated by immunomagnetic beads separation methods. The separated activated T cells (which are often alloreactive) are then allostimulated and is furhter cultured in the presence the cytokines mentioned above. Hereby the alloactivated T cells are expanded resulting in an antigen specific T cell line.

This procedure may be used for any other relevant disease including the diseases mentioned in the present application.

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CLAIMS

1. A continuous antigen reactive T cell line obtained in vitro by
a) obtaining T cells and antigen presenting cells from a mammal including a human,
b) mixing or contacting said T cells and antigen presenting cells with an antigen
c) and culturing the mixed cells in a medium comprising at least one cytokine.

2. T cell line according to claim 1
wherein the cytokines of the medium are selected from the group of IL2, IL-4, IL-7, IL-9, and IL-15.

3. T cell line according to claim 2

wherein the cytokine of the medium is a combination of cytokine IL-2 and cytokine IL-4.

4. T cell line according any of claim 1-3 wherein the T cells and antigen presenting cells are obtained as a biopsy or as a blood sample.

5. Method for preparing an antigen reactive continuous T cell line comprising
a) obtaining T cells and antigen presenting cells from a mammal including a human,
b) mixing/contacting said T cells and antigen presenting cells with an antigen
c) and culturing the mixed cells in a medium comprising at least one cytokine.

6. Method for preparing an antigen reactive continuous T cell line comprising [illegible]senting cells from a mammal includin[illegible]ssociated antigen or a superantigen and culturing the T cells in a medium comprising at least one cytokine.

7. Method according to any of claims 5 and 6 wherein the cytokines are selected from the group of IL2, IL-4, IL-7, IL-9, and IL-15.

8. Method according to any of claims 5-7 wherein the cytokine is a combination of cytokine IL-2 and cytokine IL-4.

9. Method according to any of claims 5-8 wherein the T cells and presenting cells is from a patient having a disease associated with the skin.

10. Method according to any of claims 5-8 wherein the T cells and presenting cells is from a patient having a disease associated with the intestines.

11. Method according to claim 10 wherein the disease is an inflammatory bowel disease.

12. Method according to any of claims 5-11 wherein the T cells and presenting cells is from a patient having a chronic inflammatory disease.

13. Method according to any of claims 5-11 wherein the T cells and presenting cells is from a patient having an auto-immune disease.

14. Method according to any of claims 5-11 wherein the T cells and presenting cells is from a patient having a malignant disease.

15. Method for treating or preventing a T cell mediated disease in a patient suffering or previously suffering from said T cell mediated disease comprising

a) preparing a continuous T cell line by use of T cells obtained from said patient and culturing said T cell with at least one cytokine,
b) preparing a T cell vaccine from the continuous T cell line
c) administering said T cell vaccine to the patient.

16. Method according to claim 15 wherein the cytokines are selected from the group of IL-2, IL-4, IL-7, IL-9 and IL-15.

17. Method according to claim 16 wherein the cytokine is a combination of IL-2 and IL-4.

18. Method according to claim 17 wherein the cytokines are used in concentrations of from 500-5000 U/ml, preferable 1000-2000 U/ml for IL-2, and concentrations of 200-1000 U/ml, such as about 500 U/ml, for IL-4.

19. Method according to any of claims 15-18 wherein the

disease of the patient is associated with the skin.

20. Method according to any of claims 15-18 wherein the disease is associated with the intestines.

21. Method according to claim 20 wherein the disease is an inflammatory bowel disease.

22. Method according to any of claims 15-21 wherein the disease is a chronic inflammatory disease.

23. Method according to any of claims 15-21 wherein the disease is an autoimmune disease.

24. Method according to any of claims 15-21 wherein the disease is a malignant disease.

25. A T cell vaccine prepared from T cells from a continuous T cell line obtained by culturing T cells isolated from a patient suffering from a T cell mediated disease in a medium supplemented with at least one cytokine.

26. Method according to claim 25 wherein the cytokines are selected from the group of IL-2, IL-4, IL-7, IL-9, and IL-15.

27. Method according to claim 26 wherein the cytokine is a combination of IL-2 and IL-4.

28. Method according to any of claims 25-27 wherein the T cells are isolated from a patient suffering from a disease associated with the skin.

29. Method according to any of claims 25-27 wherein the T cells are isolated from a patient suffering from a disease associated with the intestines.

30. Method according to claim 29 wherein the T cells are

isolated from a patient suffering from an inflammatory bowel disease.

31. Method according to any of claims 25-27 wherein the T cells are isolated from a patient suffering from a chronic inflammatory disease.

32. Method according to any of claims 25-27 wherein the T cells are isolated from a patient suffering from an autoimmune disease.

33. Method according to any of claims 25-27 wherein the T cells are isolated from a patient suffering from a malignant disease.

34. Method for preparing antigen activated T cell line comprising
a) obtaining a cell sample from a patient
b) culturing the cells in a medium comprising at least one cytokine
c) separating activated T cell
d) stimulation of said separated activated T cell with an allogen.
e) further culturing the allostimulated T cell in a medium comprising at least one cytokine for the production of a continuos cell line.

35. Method according to claim 34 wherein the cytokines are selected from the group of IL-2, IL-4, IL-7, IL-9, and IL-15.

36. Method according to any of claims 34 and 35 wherein the cytokine is a combination of IL-2 and IL-4.

37. Method according to any of claims 34-36 wherein the T cells are isolated from a patient suffering from a disease associated with the skin.

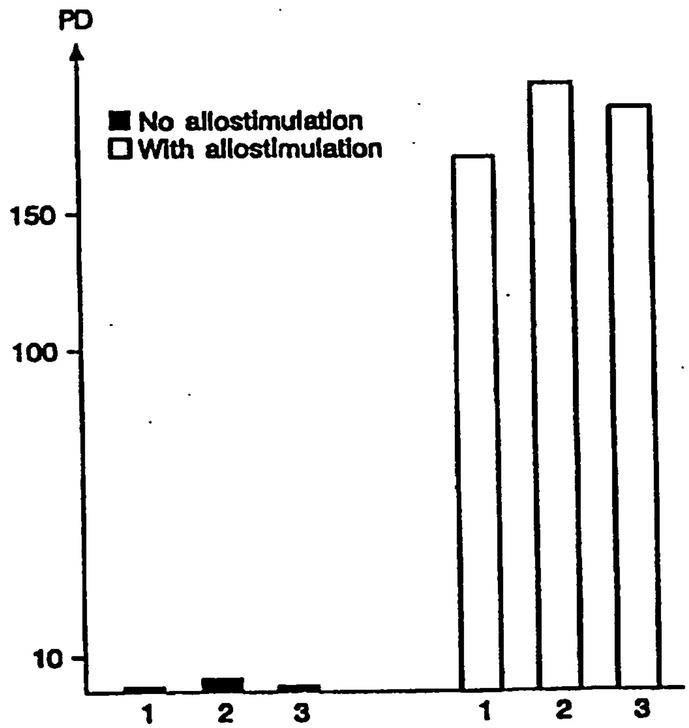
38. Method according to any of claims 34-36 wherein the T cells are isolated from a patient suffering from a disease associated with the intestines.

39. Method according to claim 38 wherein the T cells are isolated from a patient suffering from an inflammatory bowel disease.

40. Method according to any of claims 34-36 wherein the T cells are isolated from a patient suffering from a chronic inflammatory disease.

41. Method according to any of claims 34-36 wherein the T cells are isolated from a patient suffering from an autoimmune disease.

42. Method according to any of claims 34-36 wherein the T cells are isolated from a patient suffering from a malignant disease.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With allostimulation
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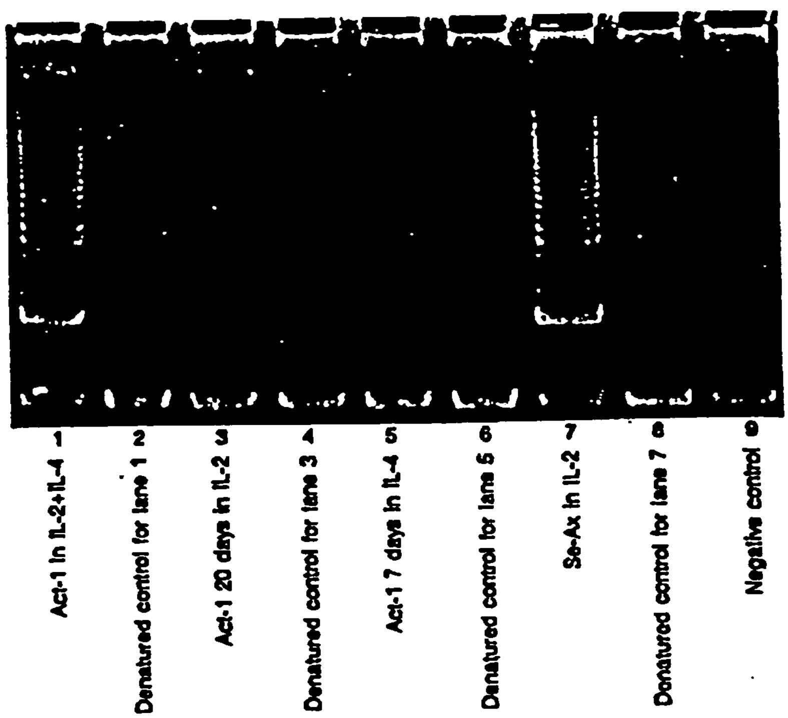
100
10
1
2
3
1
2
3

Fig. 1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2 Denatured control for lane 1
3 Act-1 20 days in IL-2
4 Denatured control for lane 3
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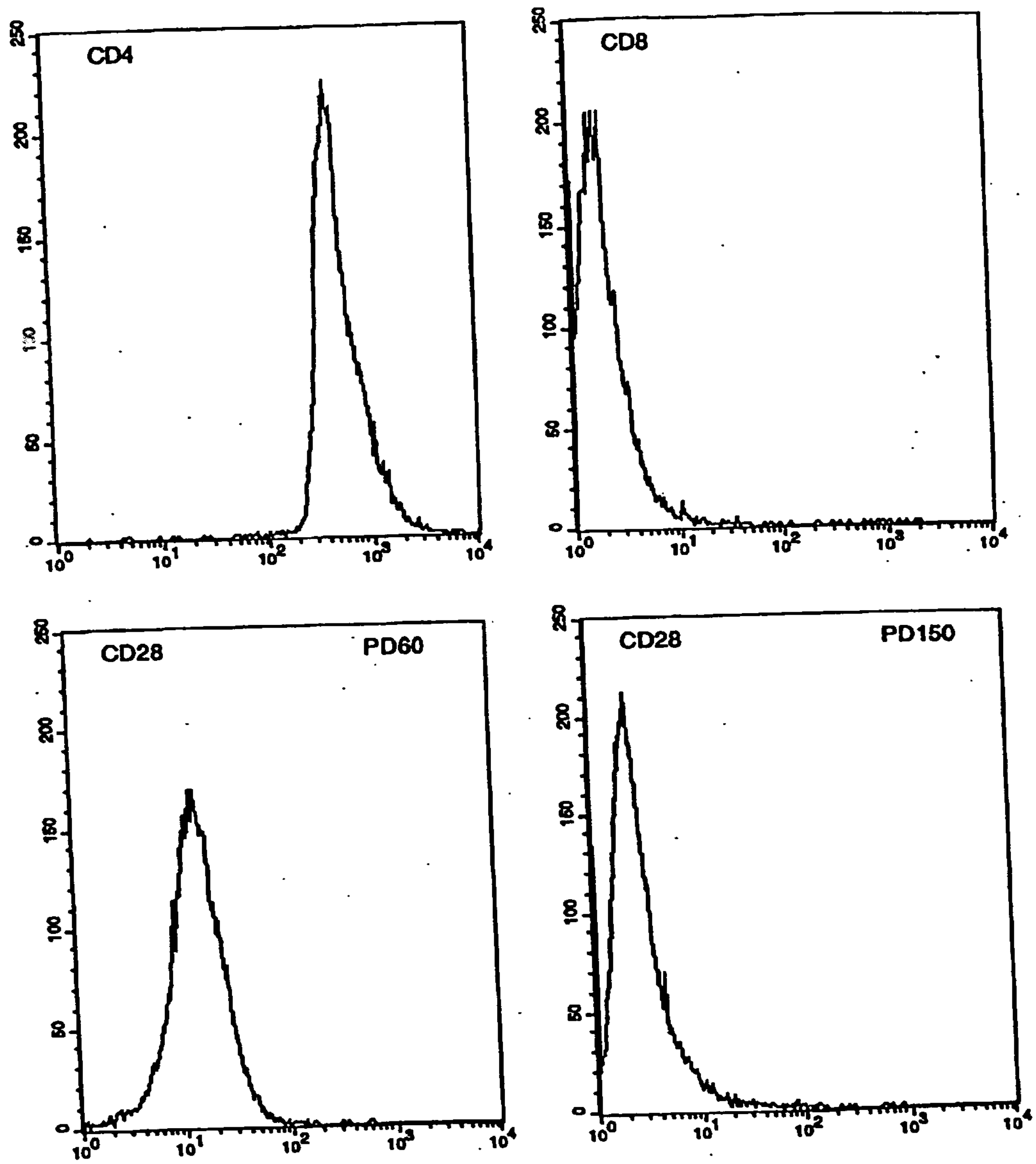
6 Denatured control for lane 5
7 Se-Ax in IL-2
8 Denatured control for lane 7
9 Negative control

Fig. 2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10^4

Fig. 3

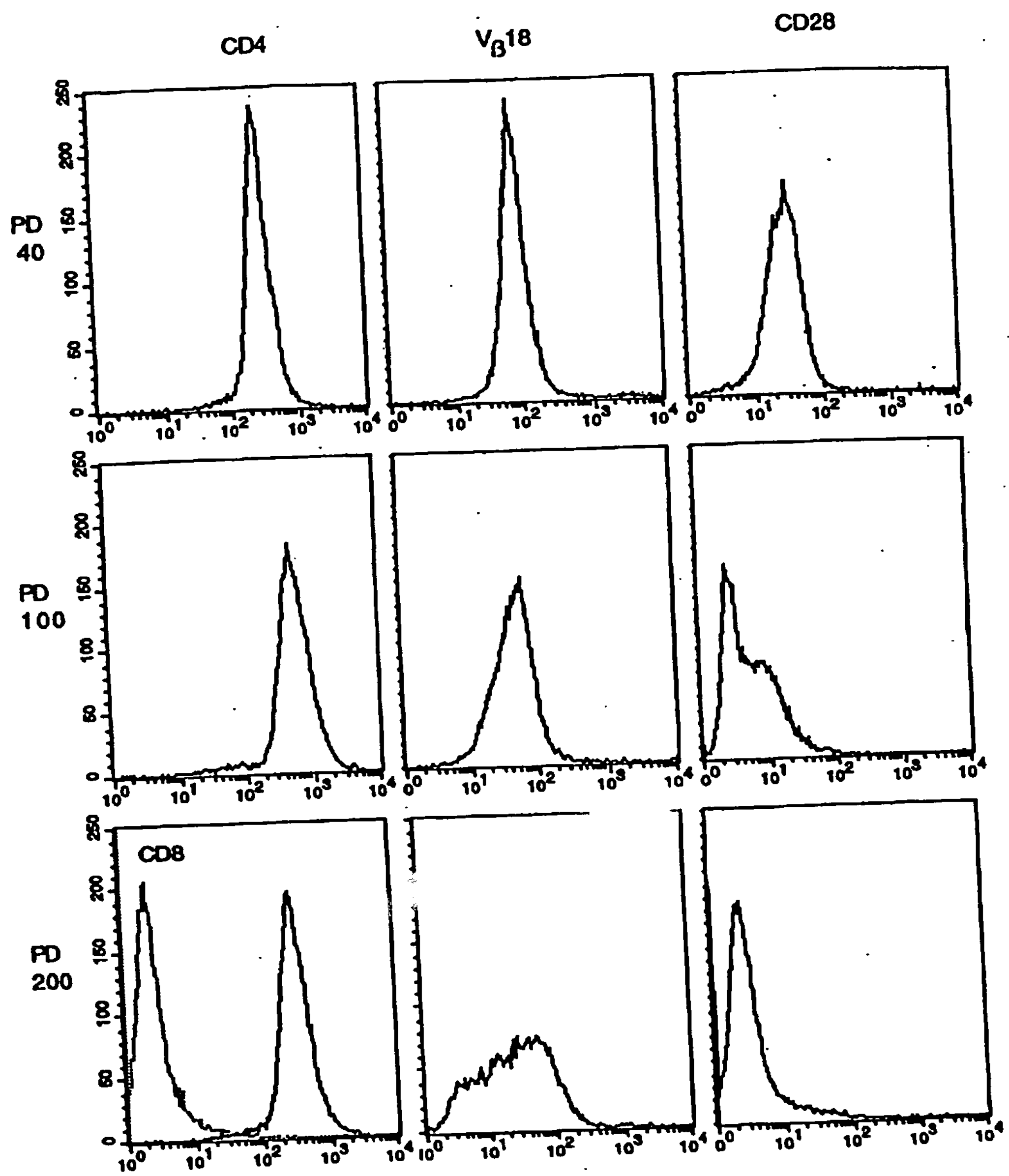

Fig. 4

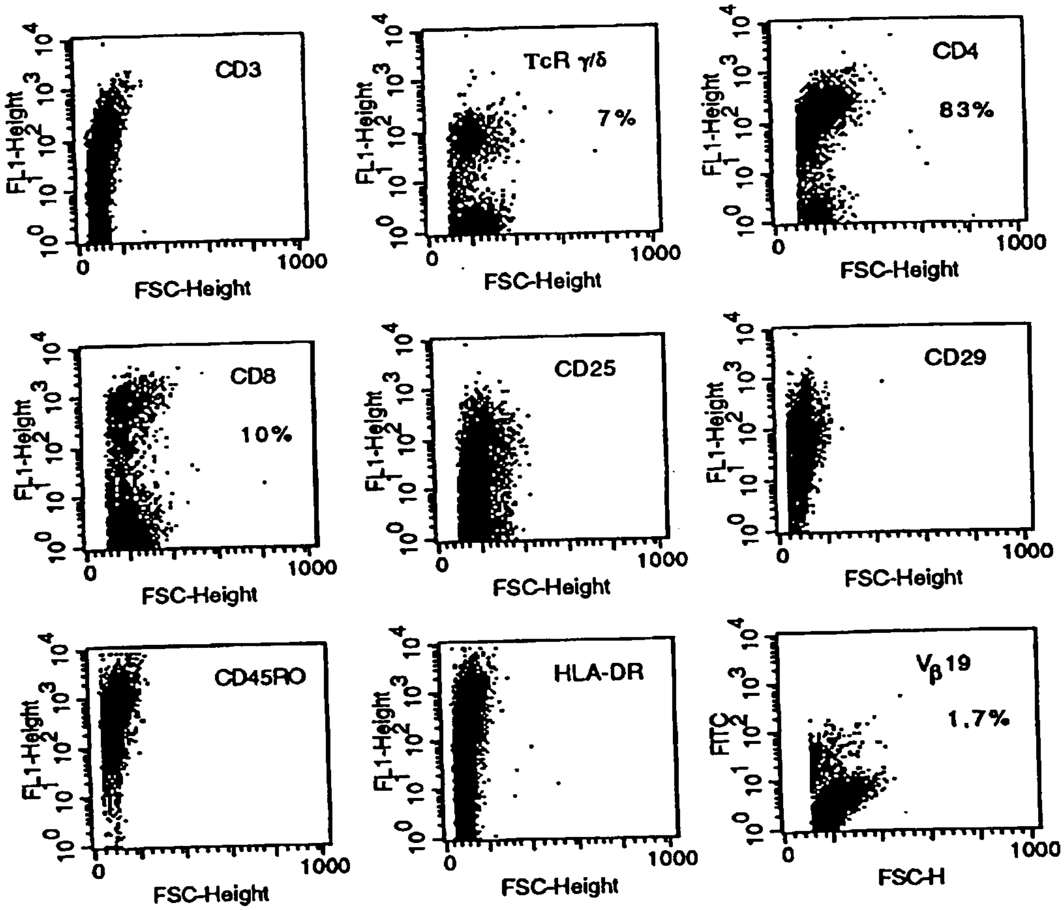
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Fig. 5

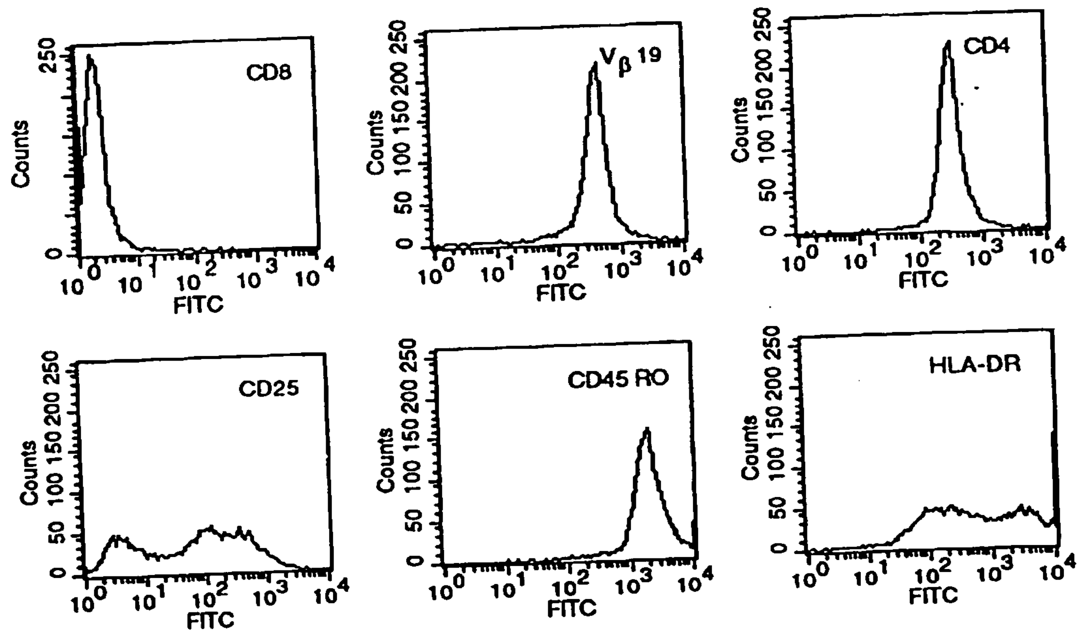
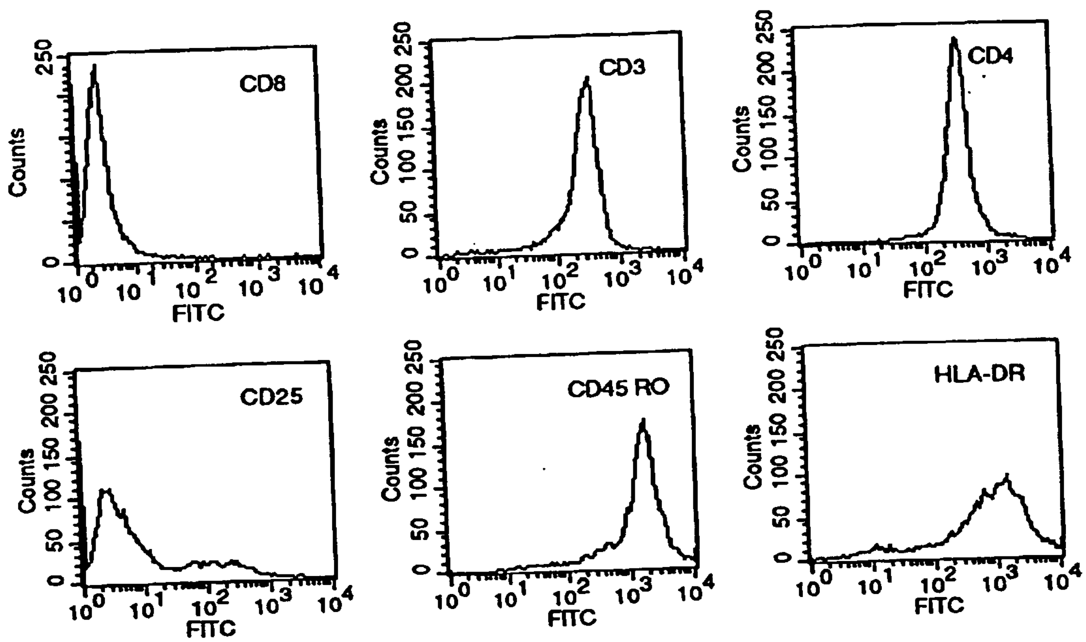


Fig. 6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CD4
CD25
CD45 RO
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10^2
10^3
10^4

Fig. 7

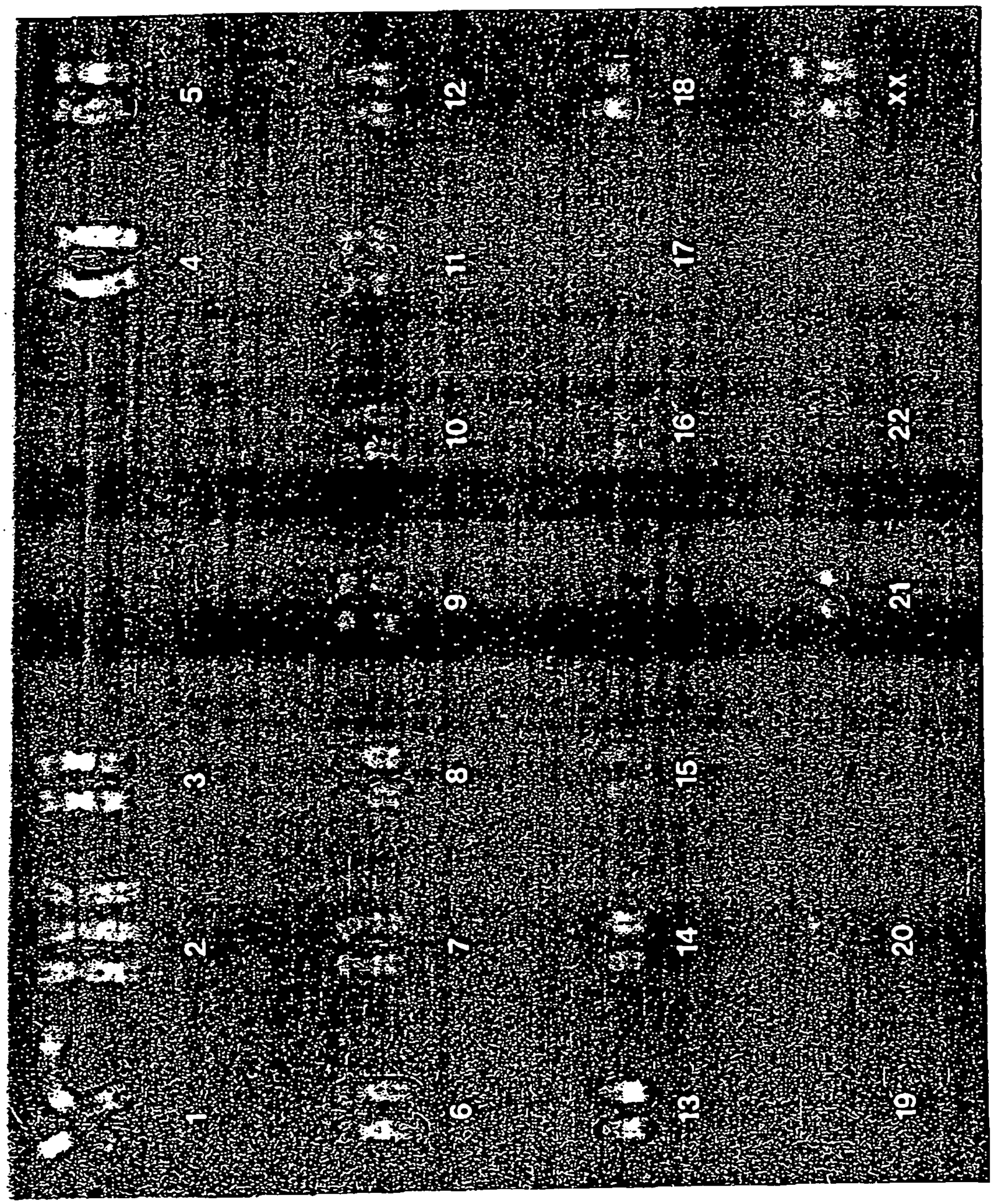

Fig. 8

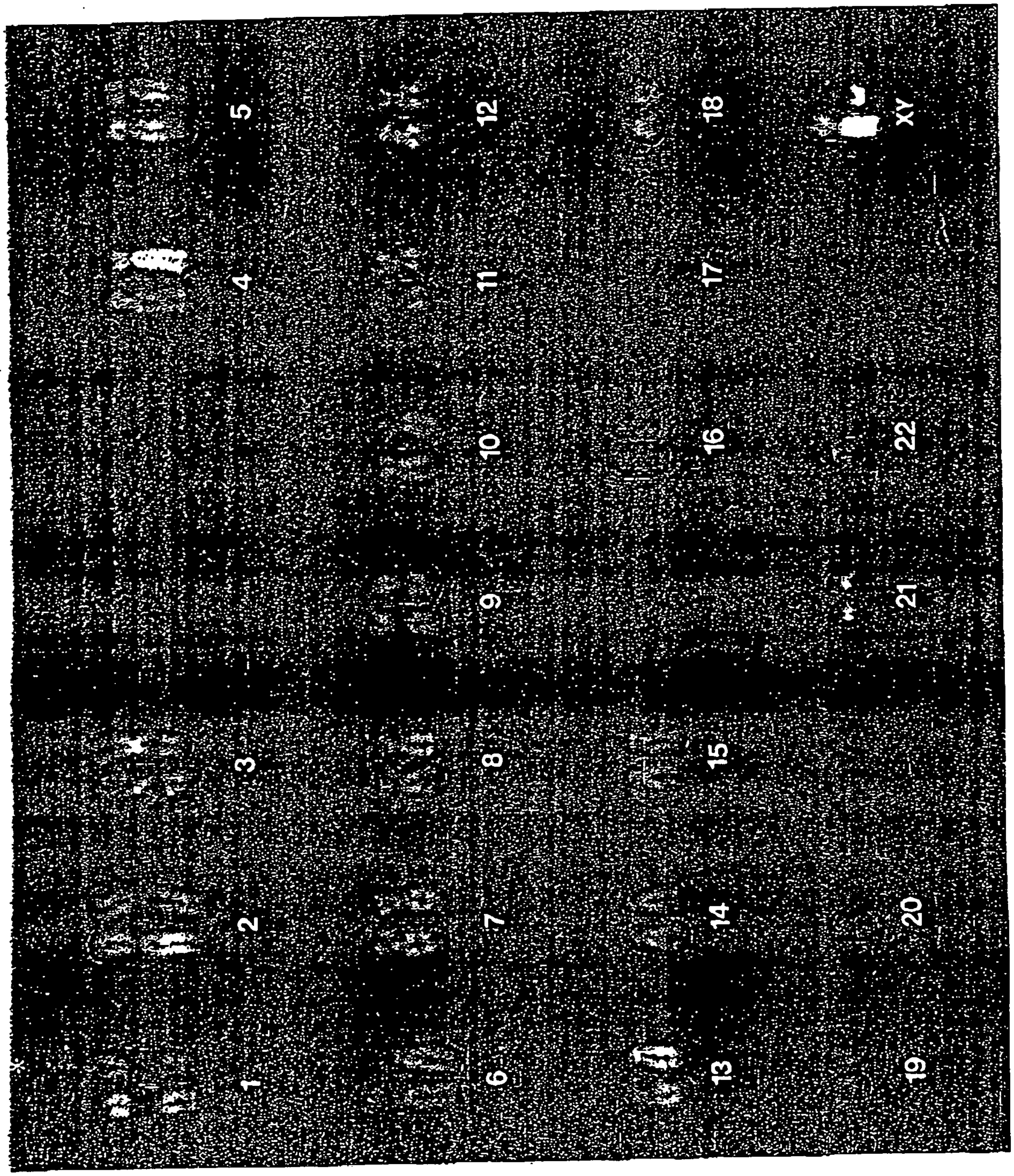

Fig. 9